Epidemiol. Infect. (2002), **128**, 337–342. © 2002 Cambridge University Press
DOI: 10.1017/S0950268801006744 Printed in the United Kingdom

SHORT REPORT
Pyrazinamide resistance associated with pncA gene mutation in *Mycobacterium tuberculosis* in Japan

T. ENDOH[1], A. YAGIHASHI[2], N. UEHARA[1], D. KOBAYASHI[2], N. TSUJI[2], M. NAKAMURA[2], S. HAYASHI[3], N. FUJII[3] AND N. WATANABE[2]*

[1] *Division of Laboratory Diagnosis, Sapporo Medical University School of Medicine, Sapporo, Japan*
[2] *Department of Clinical Laboratory Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan*
[3] *Department of Microbiology, Sapporo Medical University School of Medicine, Sapporo, Japan*

(*Accepted 25 July 2001*)

SUMMARY

Thirty Japanese clinical isolates of *Mycobacterium tuberculosis* were analysed by pyrazinamide susceptibility testing and pyrazinamidase assay, as well as polymerase chain reaction for single-strand conformational polymorphism and direct sequencing of the gene encoding pyrazinamidase (pncA). All sensitive isolates showed pyrazinamidase activity and a wild-type pncA gene, but three resistant isolates had pncA gene mutations and lacked pyrazinamidase activity. The latter isolates showed a minimum inhibitory concentration of at least 100 mg/l by the 7H10 agar proportion method and 400 mg/l by the 7H9 liquid medium method. Isolate 28 showed T-to-C change at position 11, leading to Leu4→Ser substitution; isolate 29 had an 8-bp deletion from position 382; and isolate 30 had A-to-C change at position 29, leading to Gln10→Pro substitution. The deletion has not been described previously. This is the first demonstration of pncA gene mutations in PZA-resistant *M. tuberculosis* strains isolated from Japanese patients.

Pyrazinamide (PZA) is an important first-line drug for short-course chemotherapy against *Mycobacterium tuberculosis*. PZA is a prodrug whose activity requires amide hydrolysis to pyrazinoic acid (POA) by the mycobacterial enzyme, pyrazinamidase (PZAse). A recent report suggests that POA may act mainly against fatty acid synthetase (FAS) I, a eukaryotic-like enzyme present in *M. tuberculosis* [1]. PZAse is encoded by the pncA gene. PZA-resistant *M. tuberculosis* strains show loss of PZAse activity, correspondingly, the pncA gene, which was cloned and characterized by Scorpio et al., reportedly shows mutations [2].

In Japan, a short course of *M. tuberculosis* chemotherapy including PZA was approved in 1996 by the Ministry of Health and Welfare after the World Health Organization recommended PZA for directly observed therapy for a short course (DOTS) in 1991 [3]. However, little is known about pncA gene mutations of *M. tuberculosis* strains in Japan [4]. We therefore studied pncA gene mutations in 30 Japanese clinical isolates of *M. tuberculosis* including some resistant to PZA.

All clinical isolates were identified by DAN–DNA hybridization assay [5] using a commercially available kit (DDH Mycobacteria; Kyokuto Pharmaceuticals, Tokyo, Japan) according to the manufacturer's instructions. *M. tuberculosis* strain ATCC35828 (with a single G nucleotide absent at position 288 in the pncA gene), a PZA-sensitive *M. tuberculosis* strain ATCC27294 (H37Rv), and a PZA-resistant *M. bovis* (BCG) strain ATCC 27289 (with a change from C to G at nucleotide position 169 in the

* Author for correspondence: Department of Clinical Laboratory Medicine, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo 060-8543, Japan.

Table 1. *Primers for amplification of the pncA gene**

Primer	Sequence (5′→3′)	Position
Primer set P1	F GTCGGTCATGTTCGCGATCG	−102 ~ 110
	R TCGGCCAGGTAGTCGCTGAT	
Primer set P2	F ATCAGCGACTACCTGGCCGA	91 ~ 270
	R GATTGCCGACGTGTCCAGAC	
Primer set P3	F TGCTGGACACGTCGGCAATC	251 ~ 420
	R GCGCACACAATGATCGGTGG	
Primer set P4	F CCACCGATCATTGTGTGCGC	401 ~ +55
	R TGCTTGCGGCGAGCGCTCCA	

F, forward primer; R, reverse primer.
* GenBank accession number AL02189.

pncA gene) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). These three strains were used as a negative and positive controls for mutations in the pncA gene.

PZA susceptibility testing was performed by the conventional agar proportion method [6] using Middlebrook 7H10 agar with oleic acid-albumin-dextrose-catalase (OADC) enrichment (pH 5·8; Difco Laboratories, Detroit, MI) and a liquid medium method [7] using Middlebrook 7H9 medium with albumin-dextrose-catalase (ADC) enrichment, (pH 5·6; Difco). Susceptibility to PZA was defined in terms of the minimum inhibitory concentration (MIC), the lowest PZA concentration able to inhibit more than 99% of growth observed on the PZA-free control. To determine MIC, 7H10 agars containing 25 mg/l, 50 mg/l, and 100 mg/l of PZA or 7H9 liquid medium containing 100 mg/l and 400 mg/l of PZA were used. PZAse activity was assayed according to the method described by Wayne [8], using a tube of 7H10 agar containing 100 mg/l of PZA and 2 g/l sodium pyruvate. Each isolate was compared to the known PZAse-positive strain (PZA-sensitive *M. tuberculosis* ATCC27297, H37Rv) and the negative strains (PZA-resistant *M. tuberculosis* ATCC35828 and *M. bovis* ATCC27289). Among the 30 clinical isolates, 25 were sensitive to PZA (MIC less than 25 mg/l) and 5 were resistant (MIC 50 mg/l or more) by the agar proportion method; 24 were sensitive to PZA (MIC less than 100 mg/l) and 6 were resistant (MIC at least 100 mg/l) by the liquid medium method. Three clinical isolates (nos 28–30) had an MIC of at least 100 mg/l by the agar proportion method and also an MIC of 400 mg/l by the liquid medium method. PZAse activity was not detected in these three isolates; the other 27 isolates were PZAse-positive.

In all 30 clinical isolates and in the control strains, polymerase chain reaction (PCR)-single conformation polymorphism (SSCP) analysis was performed for rapid detection of mutations in the pncA gene. The pncA gene was amplified as four separate partial sequences to optimize detection sensitivity by SSCP (all amplified PCR products had fewer than 200 bp) [9]. Two sets of primers were described previously [10], while two other sets of primers were constructed to amplify fragments of the pncA gene extending from positions −102 to 110, and from 401 to +55 based upon the GenBank sequence (accession number AL02189; Table 1). The PCR mixture contained the following reagents: 1×PCR buffer, 0·25 mM of dNTPs, 5 pmol of primers, 2·5-mM concentration of $MgCl_2$, 10 μg of genomic DNA, and 2·5 U of Taq DNA polymerase. PCR conditions programmed with an Applied Biosystems model 9600 thermal cycler (Foster, CA) were 95 °C for 5 min followed by 30 cycles at 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec. SSCP analysis was performed according to a modified method described by Scorpio et al. [10]. Briefly, 0·5 to 1·0 μg of PCR products were denatured with 9 volumes of formamide dye solution (95% formamide, 10 mM sodium hydroxide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0·05% bromophenol blue, and 0·05% xylene cyanol FF) at 95 °C, and then cooled and maintained on ice. Denatured PCR products were loaded onto a 6% polyacrylamide gel containing 5% glycerol (16 by 20 cm; acrylamide: bis-acrylamide, 49:1). Electrophoresis was performed in 0·5×TBE buffer containing 0·05 M Tris, 0·045 M Boric acid, 0·0005 M EDTA at a constant voltage of 150 V (5 h, 20 °C). Bands on the gels were visualized by silver staining method (Plus One DNA silver staining kit, Amersham Pharmacia Biotech, Uppsala, Sweden). SSCP band patterns obtained were compared with those of control strains. SSCP band

https://doi.org/10.1017/S0950268801006744 Published online by Cambridge University Press

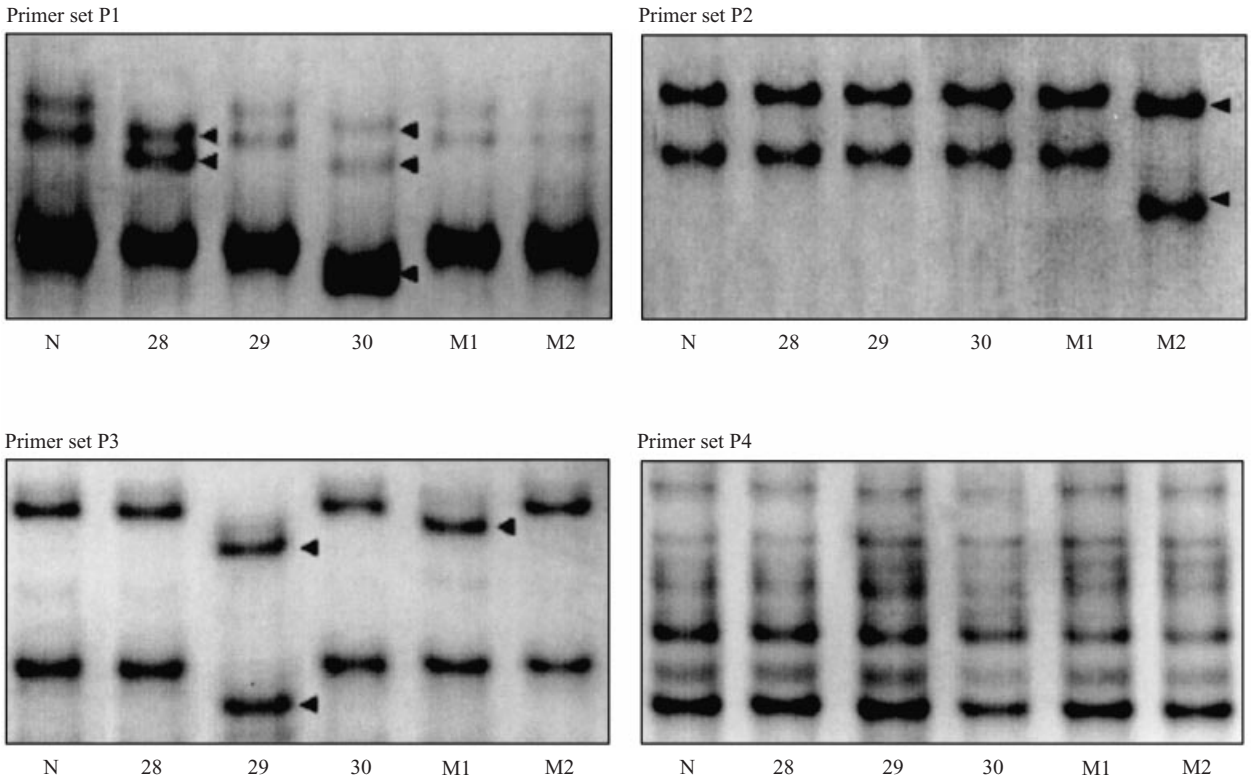

Fig. 1. Polymerase chain reaction-single strand conformation polymorphism (PCR–SSCP) analysis of pncA gene mutations in *Mycobacterium tuberculosis* isolates. Primer sets used are described in Table 1. Lane N, pyrazinamide (PZA)-sensitive *M. tuberculosis* ATCC27294; lanes 28–30, PZA-resistant clinical *M. tuberculosis* isolate nos 28–30; lanes M1 and 2, PZA-resistant *M. tuberculosis* ATCC35828 and ATCC27289.

patterns of clinical *M. tuberculosis* isolate no. 28 (in the primer set P1 region), no. 29 (in the primer set P3 region), and no. 30 (in the primer set P1 region) differed from those of PZA-susceptible *M. tuberculosis* strain ATCC27294 (H37Rv), as show in Figure 1. Other clinical isolates did not show any difference in SSCP band patterns compared with PZA-sensitive *M. tuberculosis* strain ATCC27294 (H37Rv). The positive control strains for pncA gene mutation, ATCC27289 (with a change from C to G at position 169) and ATCC35828 (with a single nucleotide deleted at position 288) had SSCP band patterns that differed from that of the positive control strain in the primer set P2 region (from position 91–270) and in the primer set P3 region (position 251–420), respectively (Fig. 1).

In addition, PCR products amplified using same pncA primers as for PCR-SSCP were cut from the gel and purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA). Sequencing reactions were performed with a DNA sequencing kit (BigDye terminator cycle sequencing Ready Reaction; Applied Biosystems) using 10 ng of purified PCR products and 3·2 pmol of primers. After products were purified by Centri-sep spin columns (Applied Biosystems), the purified products were directly sequenced using a genetic analyzer (ABI PRISM 310; Applied Biosystems). The sequences were compared with the published sequence of pncA using sequence similarity search software (Basic Local Alignment Search Tool, or BLAST) from the National Center for Biotechnology Information (NCBI, Bethesda, MD). Results of PCR-direct sequence analysis of pncA gene are shown in Table 2. Clinical isolate no. 28 had a T-to-C change at position 11, leading to a Leu4→Ser amino-acid substitution; Isolate no. 29 had an 8-bp deletion at position 382; Isolate no. 30 had an A-to-C change at position 29, leading to a Gln10→Pro amino-acid substitution. These mutation positions determined by PCR-direct sequencing were consistent with the amplified regions showing abnormal SSCP band patterns. Mutations of the pncA gene in positive control strains were confirmed by PCR-direct sequence analysis (Table 2).

In this study, all PZA-sensitive clinical *M. tuberculosis* isolates showed PZAse activity and no mutation in the pncA gene, while three PZA-resistant

https://doi.org/10.1017/S0950268801006744 Published online by Cambridge University Press

Table 2. *Characteristics and pncA mutations in clinical* Mycobacterium tuberculosis *isolates*

Strain no.	MIC for PZA (mg/l)		PZase activity		Main associated drug resistance‡	PCR–SSCP pattern (region of abnormal pattern)	Variation of nucleotide sequence and amino-acid sequence
	Agar proportion method*	Liquid medium method†	4-days culture	7-days culture			
1	< 25	< 100	Positive	Not tested	H	Normal	
2	< 25	< 100	Positive	Not tested	E	Normal	
3	50	100	Positive	Not tested		Normal	
4	< 25	< 100	Positive	Not tested	H	Normal	
5	< 25	< 100	Positive	Not tested		Normal	
6	< 25	< 100	Positive	Not tested	H	Normal	
7	< 25	< 100	Positive	Not tested		Normal	
8	< 25	< 100	Positive	Not tested	R	Normal	
9	< 25	100	Positive	Not tested	H	Normal	
10	< 25	< 100	Positive	Not tested	H	Normal	
11	< 25	< 100	Positive	Not tested		Normal	
12	< 25	< 100	Positive	Not tested		Normal	
13	< 25	< 100	Positive	Not tested		Normal	
14	< 25	< 100	Positive	Not tested	H, R	Normal	
15	< 25	100	Positive	Not tested	H, R, S, E	Normal	
16	< 25	< 100	Positive	Not tested		Normal	
17	< 25	< 100	Positive	Not tested	H, R, S, E	Normal	
18	50	< 100	Positive	Not tested	H	Normal	
19	< 25	< 100	Positive	Not tested		Normal	
20	< 25	< 100	Positive	Not tested	S	Normal	
21	< 25	< 100	Positive	Not tested		Normal	
22	< 25	< 100	Positive	Not tested	R, S	Normal	
23	< 25	< 100	Positive	Not tested	H	Normal	
24	< 25	< 100	Positive	Not tested		Normal	
25	< 25	< 100	Positive	Not tested		Normal	
26	< 25	< 100	Positive	Not tested		Normal	
27	< 25	< 100	Positive	Not tested	S, E	Normal	
28	> 100	> 400	Negative	Negative	H, R, S, E	Abnormal (set P1)	T11→C (leu4→ser)
29	> 100	> 400	Negative	Negative	H, R, S, E	Abnormal (set P3)	8-bp deletion at position 382
30	> 100	> 400	Negative	Negative	H, R, S, E	Abnormal (set P1)	A29→C (gln10→pro)
ATCC 35828	> 100	> 400	Negative	Negative		Abnormal (set P3)	Nucleotide G deletion at position 288
ATCC 27289	> 100	> 400	Negative	Negative		Abnormal (set P2)	C169→G

* < 25 mg/l is susceptible; ≧ 50 mg/l is resistant. † < 100 mg/l is susceptible; ≧ 100 mg/l is resistant. ‡ H, isoniazid; R, rifampin; S, streptomycin; E, ethambutol. MIC, minimal inhibitory concentration; PZA, pyrazinamide; PZase, pyrazinamidase; ATCC, American Type Culture Collection; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism.

isolates (nos 28–30) had mutations in the pncA gene and no PZAse activity. These three isolates showed high MIC for PZA (MIC at least 100 mg/l by the 7H10 agar proportion method and 400 mg/l by the 7H9 liquid medium method). Glenn et al. [11] also reported that *M. tuberculosis* with a pncA gene mutation showed similarly high MICs for PZA. In addition, our three PZA resistant isolates showed resistance to several additional antituberculous drugs such as isoniazid, rifampin, streptomycin, and ethambutol. Our results are consistent with previous reports that PZA-resistant *M. tuberculosis* strains with pncA gene mutations show multiple-drug resistance.

Three of our isolates (nos 9, 15, 18) showed a discrepancy between the two MIC determinations, and were identified as PZA-resistant by only one method. In addition, our isolate no. 3 showed an MIC of 50 mg/l by the 7H10 agar proportion method and 100 mg/l by the 7H9 liquid medium method, qualifying as a PZA-resistant strain by both assays. All four isolates showed PZAse activity, and none had a mutation in the pncA gene. Notably, conventional susceptibility testing for PZA is difficult to perform because PZA is most active at a pH of approximately 5·5, which in itself, inhibits growth of *M. tuberculosis* [7, 12, 13]. These four isolates may have had important mutations apart from the pncA gene, they may have shown false resistance because of difficulties of culture under acidic conditions.

We demonstrated three different mutations of the pncA gene in three clinical *M. tuberculosis* isolates from Japanese patients: isolate no. 28 had a T-to-C change at position 11, leading to a Leu4→Ser amino-acid substitution; no. 29 had an 8-bp deletion at position 382; and no. 30 had an A-to-C change at position 29, leading to a Gln10→Pro amino-acid substitution. Although the significance of pncA gene mutations in *M. tuberculosis* is not altogether clear, a single-nucleotide mutation was noted frequently in PZA-resistant *M. tuberculosis* in previous reports. Approximately 40% of distinct amino acid substitutions involved replacement with a proline residue. Insertion or deletion of multiple nucleotides tended to be found in the downstream portion of the pncA gene [2, 10, 11, 14–20]. In our three clinical PZA-resistant *M. tuberculosis* isolates with pncA gene mutations, two had single-nucleotide mutations (Leu4 to Ser or Gln10 to Pro), while one had a deletion in the downstream portion of the pncA gene that has not been described previously. This deletion is close to the regions (Ala-134, Thr-135, and Cys-138) which is considered the key residues for hydrolysis of PZA and resulted in frameshift [21].

Davies et al. concluded that pncA genotype determinations in PZA-resistant *M. tuberculosis* by methods such as PCR-SSCP and PCR-direct sequencing cannot reliably determine the mechanism of resistance, because these examine only one possible cause [22]. A PZA-resistant *M. tuberculosis* isolates does not necessarily have mutations in the pncA gene; the frequency of pncA gene mutation has been estimated to be 72–97% in PZA-resistant clinical isolates [2, 10, 18, 19]. In addition, culture methods may induce mutations in *M. tuberculosis*; Scorpio et al. succeeded in inducing a mutation using 7H11 agar with 500 mg/l of PZA [10].

Therefore, genotype determinations must be combined with phenotyping of PZA-resistant *M. tuberculosis*, such as PZAse activity assay and pyrazinamide susceptibility testing.

Until now, the brief history of antituberculous chemotherapy with PZA in Japan has included no reports concerning PZA resistance and pncA gene mutations. This is the first demonstration of pncA gene mutations in PZA-resistant *M. tuberculosis* strains isolated from Japanese patients. In addition, the deletion found in isolate no. 29 has not been described previously.

REFERENCES

1. Zimhony O, Cox JS, Welch JT, Vilcheze C, Jacobs WR Jr. Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FAS I) of *Mycobacterium tuberculosis*. Nat Med 2000; **6**: 1043–7.
2. Scorpio A, Zhang Y. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med 1996; **2**: 662–7.
3. World Health Organization. WHO report on the Tuberculosis Epidemic Stop TB at the source. Tuberculosis Programme, World Health Organization, Geneva, Switzerland, 1995.
4. Hoashi S, Tai H, Tamari M. PncA gene mutations in clinical isolates of tubercle bacillus by polymerase chain reaction-direct sequencing method: in relationship to pyrazinamide resistance. Kekkaku 1999; **74**: 441–5.
5. Kusunoki S, Ezaki T, Tamesada M, Hatanaka Y, Asano K, Hashimoto Y, Yabuuchi E. Application of colorimetric microdilution plate hybridization for rapid genetic identification of 22 *Mycobacterium* species. J Clin Microbiol 1991; **29**: 1596–603.
6. Butler WR, Kilburn JO. Improved method for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. J Clin Microbiol 1982; **16**: 1106–9.

https://doi.org/10.1017/S0950268801006744 Published online by Cambridge University Press

7. McDermott W, Tomsett R. Activation of pyradinamide and nicotinamide in acidic environment in vitro. Am Rev Tuberc 1954; **70**: 748–54.
8. Wayne LG. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis 1974; **109**: 147–51.
9. Ainsworth PJ, Surh LC, Coulter-Mackie MB. Diagnostic single strand conformational polymorphism, (SSCP): a simplified non-radio isotopic method as applied to a Tay-Sachs B1 variant. Nucl Acids Res 1992; **19**: 405–6.
10. Scorpio A, Lindholm-Levy P, Heifets L, et al. Characterization of pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 1997; **41**: 540–3.
11. Morlock GP, Crawford JT, Butler WR, et al. Phenotypic characterization of pncA mutants of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2000; **44**: 2291–5.
12. Heifets L, Lindholm-Levy P. Pyrazinamide sterilizing activity in vitro against semidormant *Mycobacterium tuberculosis* bacterial populations. Am Rev Respir Dis 1992; **145**: 1223–5.
13. Stottmeier KD, Beam RE, Kubica GP. Determination of drug susceptibility of mycobacteria to pyrazinamide in 7H10 agar. Am Rev Respir Dis 1967; **96**: 1072–5.
14. Hou L, Osei-Hyiaman D, Zhang Z, Wang B, Yang A, Kano K. Molecular characterization of pncA gene mutations in *Mycobacterium tuberculosis* clinical isolates from China. Epidemiol Infect 2000; **124**: 227–32.
15. Cheng SJ, Thibert L, Sanchez T, Heifets L, Zhang Y. pncA mutations as a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*: spread of a monoresistant strain in Quebec, Canada. Antimicrob Agents Chemother 2000; **44**: 528–32.
16. Escalante P, Ramaswamy S, Sanabria H, et al. Genotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Peru. Tuber Lung Dis 1998; **79**: 111–8.
17. Marttila HJ, Marjamaki M, Vyshnevskaya E, et al. PncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* isolates from northwestern Russia. Antimicrob Agents Chemother 1999; **43**: 1764–6.
18. Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C. Mutation in pncA is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuber Lung Dis 1997; **78**: 117–22.
19. Sreevatsan S, Pan X, Zhang Y, Kreiswirth BN, Musser JM. Mutations associated with pyrazinamide resistance in pncA of *Mycobacterium tuberculosis* complex organisms. Antimicrob Agents Chemother 1997; **41**: 636–40.
20. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tubercle Lung Dis 1998; **79**: 3–29.
21. Lemaitre N, Callebaut I, Frenois F, Jarlier V, Sougakoff W. Study of the structure-activity relationships for the pyrazinamidase (PncA) from *Mycobacterium tuberculosis*. Biochem J 2001; **353**: 453–8.
22. Davies AP, Billington OJ, McHugh TD, Mitchison DA, Gillespie SH. Comparison of phenotypic and genotypic methods for pyrazinamide susceptibility testing with mycobacterium tuberculosis. J Clin Microbiol 2000; **38**: 3686–8.